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Citation for published version:

Cook, IH, Evans, J, Maldonado-Perez, D, Critchley, HO, Sales, KJ & Jabbour, HN 2010, 'Prokineticin-1 (PROK1) modulates interleukin (IL)-11 expression via prokineticin receptor 1 (PROKR1) and the calcineurin/NFAT signalling pathway', *Molecular Human Reproduction*, vol. 16, no. 3, pp. 158-169.
<https://doi.org/10.1093/molehr/gap084>

Digital Object Identifier (DOI):

[10.1093/molehr/gap084](https://doi.org/10.1093/molehr/gap084)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Molecular Human Reproduction

Publisher Rights Statement:

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Prokineticin-I (PROK1) modulates interleukin (IL)-11 expression via prokineticin receptor 1 (PROKR1) and the calcineurin/NFAT signalling pathway

Ian H. Cook¹, Jemma Evans¹, David Maldonado-Pérez^{1,2}, Hilary O. Critchley², Kurt J. Sales¹, and Henry N. Jabbour^{1,3}

¹Medical Research Council, Human Reproductive Sciences Unit, University of Edinburgh, Edinburgh EH16 4TJ, UK ²Department of Reproductive and Developmental Sciences, University of Edinburgh, Edinburgh EH16 4TJ, UK

³Correspondence address. Human Reproductive Sciences Unit, Medical Research Council, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, UK. Tel: +44-131-2426220; Fax: +44-131-2426231; Email: h.jabbour@hrc.ac.uk

ABSTRACT: Prokineticin-I (PROK1) is a multifunctional secreted protein which signals via the G-protein coupled receptor, PROKR1. Previous data from our laboratory using a human genome survey microarray showed that PROK1–prokineticin receptor 1 (PROKR1) signalling regulates numerous genes important for establishment of early pregnancy, including the cytokine interleukin (IL)-11. Here, we have shown that PROK1–PROKR1 induces the expression of IL-11 in PROKR1 Ishikawa cells and first trimester decidua via the calcium–calcineurin signalling pathway in a guanine nucleotide-binding protein ($G_{q/11}$), extracellular signal-regulated kinases, Ca^{2+} and calcineurin–nuclear factor of activated T cells dependent manner. Conversely, treatment of human decidua with a lentiviral miRNA to abolish endogenous PROK1 expression results in a significant reduction in IL-11 expression and secretion. Importantly, we have also shown a regulatory role for the regulator of calcineurin 1 isoform 4 (RCAN1-4). Overexpression of RCAN1-4 in PROKR1 Ishikawa cells using an adenovirus leads to a reduction in PROK1 induced IL-11 indicating that RCAN1-4 is a negative regulator in the calcineurin-mediated signalling to IL-11. Finally, we have shown the potential for both autocrine and paracrine signalling in the human endometrium by co-localizing IL-11, IL-11R α and PROKR1 within the stromal and glandular epithelial cells of non-pregnant endometrium and first trimester decidua. Overall we have identified and characterized the signalling components of a novel PROK1–PROKR1 signalling pathway regulating IL-11.

Key words: endometrium / interleukin-11 / prokineticin-1 / RCAN1-4

Introduction

The prokineticins (PROK) are a family of multifunctional secreted proteins consisting of two members called prokineticin 1 (PROK1), also known as endocrine gland vascular endothelial growth factor, EG-VEGF (LeCouter *et al.*, 2001; Li *et al.*, 2001) and PROK2, also known as Bombina variegata 8, Bv8 (Mollay *et al.*, 1999). Prokineticins have been shown to regulate angiogenesis (Urayama *et al.*, 2007), haematopoiesis (LeCouter *et al.*, 2004), intestinal contraction (Li *et al.*, 2001), neurogenesis (Ng *et al.*, 2005) and pain sensation (Negri *et al.*, 2006). Prokineticins bind to two closely related G-protein coupled receptors, known as prokineticin receptor 1 (PROKR1) and prokineticin receptor 2 (PROKR2), with both

receptors able to bind PROK1 and PROK2 with similar affinities (Soga *et al.*, 2002). Prokineticins and their receptors are expressed in the male and female reproductive tracts (reviewed in Maldonado-Perez *et al.*, 2007). PROK1 and PROKR1 show differential expression across the menstrual cycle and in first trimester decidua, with increased endometrial expression of PROK1 observed in the mid-secretory phase and both PROK1 and PROKR1 increased in first trimester decidua. PROK1 and PROKR1 immunolocalize to stromal, endothelial and glandular epithelial cells of the endometrium and smooth muscle and endothelial cells in the myometrium (Battersby *et al.*, 2004; Evans *et al.*, 2008). Recent data from our laboratory demonstrated that PROK1–PROKR1 signalling regulates genes important for the establishment of early pregnancy. These genes included

leukemia inhibitory factor (LIF), cyclooxygenase (COX)-2, interleukin-8 and interleukin-11 (IL-11) (Evans *et al.*, 2008, 2009).

In the present study, we investigated the role of PROK1–PROKR1 modulation of IL-11. IL-11 is a member of the GPI30 family of cytokines which includes LIF, interleukin-6 and cardiotrophin 1 (Dimitriadis *et al.*, 2005a, b). IL-11 signals initially by binding to its low affinity receptor IL-11R α which subsequently recruits and binds the GPI30 subunit, dimerizes and forms an active high affinity complex (Bilinski *et al.*, 1998). Signalling from IL-11R α –GPI30 is often via the mitogen-activated protein kinase signalling pathways (Yin and Yang, 1994; Yang and Yin, 1995).

IL-11 is essential for mouse implantation. Female mice with a null mutation in IL-11R α are infertile due to poor decidualization resulting in over invasiveness of the implanting blastocyst (Bilinski *et al.*, 1998; Robb *et al.*, 1998). Further studies using the IL-11R α mice have elucidated a role for IL-11 in the differentiation of uterine natural killer (uNK) cells at the maternal–fetal interface (Ain *et al.*, 2004). In human endometrial stromal cells, IL-11 has been shown to advance progesterone-induced decidualization implying a role for IL-11 in preparing the endometrium for implantation (Dimitriadis *et al.*, 2002). Furthermore relaxin and prostaglandin E₂ (PGE₂) have both been shown to increase IL-11 mRNA and protein secretion in decidualized endometrial stromal cells (Dimitriadis *et al.*, 2005a, b). Finally, Karpovich *et al.* (2005) have shown that decidualized human endometrial stromal cells taken from patients with primary infertility produce lower levels of IL-11 compared with cells derived from fertile women indicating a potential role for IL-11 in decidualization and successful pregnancy.

In this study, we have confirmed our initial human genome survey microarray observation that PROK1 regulates IL-11 expression (Evans *et al.*, 2008). Importantly in the present study, we have mapped out the cellular and molecular pathways regulating IL-11 via PROK1 in endometrium and first trimester decidua. We found that PROK1–PROKR1 regulates IL-11 via the calcineurin signalling pathway in a guanine nucleotide-binding protein (G_{q/11}), calcium and extracellular signal-regulated kinase (ERK) dependent manner in human endometrium and first trimester decidua. We confirmed the role of PROK1 in regulating basal IL-11 expression in first trimester decidua by using a miRNA targeted to PROK1. Furthermore we have shown that overexpression of regulator of calcineurin 1 isoform 4 (RCAN1-4)—a negative regulator of calcineurin signalling—leads to a reduction in PROK1 induced IL-11, indicating that RCAN1-4 is acting as a negative regulator in the signalling pathway mediating IL-11 expression. IL-11 is known to be essential for successful decidualization and implantation and our data have characterised a novel pathway that regulates IL-11 secretion via PROK1–PROKR1 in human endometrium and first trimester decidua.

Experimental procedures

Reagents

DMEM F-12 GLUTAMAX cell culture medium was purchased from Invitrogen Life Technologies (Paisley, UK). YM-254890 was kindly donated by Astellas Pharma Inc (Tsukuba, Japan). Cyclosporin A (Calcineurin inhibitor), Inhibitor of NFAT–Calcineurin Association-6 (INCA-6), PD98059 (MAPK kinase (MEK) inhibitor) and BAPTA-AM (Ca²⁺ chelator) were purchased from Calbiochem (Nottingham, UK). EGTA (Ca²⁺ chelator) was purchased from Sigma

(Dorset, UK). PROK1 was purchased from Peprotech (London, UK) and used at 40 nM in all experiments.

Patients and tissue collection

Non-pregnant endometrial tissue ($n = 44$) at different stages of the menstrual cycle was collected from women undergoing surgery for minor gynaecological procedures using an endometrial suction curette (Pipelle, Laboratoire CCD, France). Women had no underlying endometrial pathology and had regular menstrual cycles of between 25 and 35 days. None of these women had received a hormonal preparation in the 3 months preceding biopsy collection. Biopsies were dated according to stated last menstrual period and confirmed by histological assessment according to criteria of Noyes *et al.* (1975). First trimester decidua (7–12 weeks, $n = 25$) was collected from women undergoing elective first-trimester surgical termination of pregnancy. Ethical approval was obtained from Lothian Research Ethics Committee and written informed consent was obtained from all subjects before tissue collection.

Cell and tissue culture and treatment

Ishikawa endometrial adenocarcinoma cells were obtained from the European Collection of Cell Culture (Wiltshire, UK). Stable PROKR1 transfected cells were designed and characterized as described before (Evans *et al.*, 2008). These PROKR1 Ishikawa cells were cultured in DMEM/F-12 cultured medium supplemented with 10% fetal bovine serum and a maintenance dose of 200 μ g/ml of G418 antibiotic.

Tissue explants were finely chopped with scissors and maintained in DMEM. Tissue was divided into equal portions for experimental procedures.

Cells and tissue were incubated in serum free DMEM overnight prior to treatments of PROK1 alone or in the presence of inhibitors (concentrations in figure legends) for times indicated in figure legends. Inhibitors were added 1 h prior to PROK1. Cells and tissues were harvested with conditioned media collected for enzyme linked immunosorbent assays (ELISAs) and RNA extracted for RT–PCR analysis.

Tissue was infected with lentivirus expressing PROK1 miRNA constructs for 72 h as described by Evans *et al.* (2009). Oligonucleotides encoding human PROK1 miRNA constructs were obtained from Invitrogen and inserted into the pcDNA6.2-GW/EmGFP-miR vector and used for transient transfections. These were recombined to create plenti6/V5-EmGFP-miR negative control and pLenti6/V5-EmGFP-hum-PROK1-72, -287 and -72_287 chained (Evans *et al.*, 2009). Following infection tissue and medium were harvested, and RNA or protein was extracted for RT–PCR or ELISA analysis.

Taqman quantitative RT–PCR

Total RNA was extracted from cells using Total RNA Isolation Reagent (TRI reagent) from Sigma (Poole, UK) following manufactures instructions using phase lock tubes from Eppendorf (Cambridge, UK). Quantified RNA samples were reverse transcribed and quantitative RT–PCR was performed as described before (Sales *et al.*, 2004) using the following primers and probes: IL-11: forward: 5'-CCCAGTTACCCAAGCATCCA-3'; reverse: 5'-AGACAGAGAA CAGGAATTAAATGTGT-3' and probe 5'-FAM-CCCCAGCTC

TCAGACAAATCGCCC-3'; IL-11R α : forward: 5'-CCAGCCAGAT CAGCGGTTTA-3': reverse: 5'-TGGCTATCAGCTCCTAGGACTG T-3' and probe 5'-FAM-CCACCCGCTACCTCACCTCCTACAGG-3'; GPI30 forward: 5'-CTGAATGGGCAACACACAAGTT-3': reverse: 5'-CCAGACTTCAATGTTGACAAAATACA-3' and probe 5'-FAM-CAAAGCAAAACGTGACACCCCAACC-3'. The expression of analysed genes was normalized for RNA loading using 18S ribosomal RNA. All results are expressed as relative to a positive RNA standard (cDNA obtained from a single endometrial tissue) included in all reactions.

Enzyme linked immunosorbent assay

Secreted IL-11 was quantified using an in-house ELISA. The assay was based on the two step direct sandwich ELISA, using a monoclonal antibody against human IL-11 as the capture antibody and a biotinylated antibody against human IL-11 as the detection antibody (both from R&D Systems, Oxford, UK). Briefly, plates were coated overnight at 4°C with capture antibody added at 100 μ l/well followed by 100 μ l/well of coating solution for 1 h at room temperature. The coating solution was removed and the plates stored at -20°C. Before use, the plates were washed twice in wash buffer (0.05% Tween-20, 10 mM TRIS, 0.15 M NaCl). Recombinant IL-11 standard (R&D, Oxford, UK) was diluted in ELISA buffer (4000–31.25 pg/ml) and 100 μ l of standard or conditioned media sample were added per well. Plates were incubated overnight at 4°C and then washed four times as above; before addition of anti-IL-11 detection antibody (R&D, Oxford, UK) at 100 ng/ml in ELISA buffer. Plates were again washed four times before addition of streptavidin–peroxidase (Boehringer Mannheim) at 1/2000 adding 100 μ l/well for 20 min at room temperature on a rocker. Plates were again washed four times before addition of tetramethylbenzidine (TMB) substrate. Colour change was monitored and stopped using 2N sulphuric acid. Plates were read on a plate reader at 450 nm within 10 min of quenching.

RCANI-4 adenovirus

Adenoviral RCANI-4 was produced using molecular cloning techniques as previously published in full detail (Maldonado-Perez et al., 2009). Briefly, the cDNA of RCANI-4 was cloned into a shuttle vector (pDC316) and subsequently HEK 293 cells were co-transfected with 0.5 μ g pDC316-RCANI-4 and 1.5 μ g adenoviral genomic plasmid pBHGlox E1,3 Cre. Adenoviral plaques were harvested 10–14 days later and virus released by 3 \times freeze/thaw cycles. Clonal plaques were obtained by serial dilution and infection of 80% confluent HEK 293 cells overlaid 5 h post inoculation with 0.5% SeaPlaque Agarose (FMC Corp, Rockland, ME, USA) dissolved in growth media. Plaques were picked 8–12 days later, inoculated into a T75 flask and incubated until 70–80% cytopathic effect (CPE) was observed. This first seed was inoculated into multiple flasks and harvested when CPE was apparent. RCANI-4 adenovirus was purified, concentrated, aliquoted and stored at -80°C (Vivapure AdenoPACK 100 purification kit; Sartorius AG, Goettingen, Germany). Titres were determined using the AdenoX Rapid titre kit (Clone-Tech). Yields of in excess of 1×10^{10} IFU/ml were routinely obtained.

PROKR1 Ishikawa cells were plated in 6 well plates at a density of 100 000 cells per well. After 24 h incubation, cells were washed

with PBS and 1 ml of fresh medium containing five adenovirus pfu/plated cell was added to each well. Cells were incubated for another 24 h and serum starved overnight before treatment with 40 nM PROKR1.

Immunohistochemistry

Localization of IL-11R α and GPI30/CD56/CD68 (double) and PROKR1, IL-11R α and IL-11 (triple) protein expression was investigated in endometrial and decidual tissues by immunohistochemistry. Five-micron paraffin wax-embedded tissue sections were cut and mounted onto coated slides (TESPA, Sigma). Sections were de-waxed in xylene, rehydrated in graded ethanol and washed in water. Antigen retrieval was performed by pressure cooking for 5 min in 0.01 M sodium citrate pH6. Sections were subsequently blocked for endogenous endoperoxidase (3% H₂O₂ in methanol).

For the IL-11R α -GPI30 double immunohistochemistry sections were blocked in normal donkey serum (NDS, one part serum, four parts PBS plus 5% BSA) and subsequently incubated overnight with a polyclonal anti-GPI30 antibody at 1:40 (Santa Cruz Biotechnology, Wiltshire, UK). Sections were washed and stained directly with a donkey anti-rabbit 488 fluorochrome (1:200 in NDS). Sections were washed, re-blocked in NDS and incubated overnight with a goat anti-IL-11R α antibody at 1:20 (A-13; Santa Cruz) followed by a donkey anti goat 546 fluorochrome (1:200 in PBS).

For the IL-11R α -CD56/CD68 double immunohistochemistry sections were blocked in normal goat serum (NGS, one part serum, four parts PBS plus 5% BSA) and subsequently incubated overnight with a polyclonal anti-IL-11R α antibody at 1:700 (C-20; Santa Cruz). Sections were washed, incubated in goat anti-rabbit peroxidase (1:500 in NGS for 30 min) followed by fluorochromes TSA-plus cyanide 3 (1:50 in diluent for 10 min, PerkinElmer, Applied Biosystems, Warrington, UK). Sections were washed and blocking in NGS was repeated before being incubated overnight with either a monoclonal anti-CD56 (1/1500 Dako, Denmark) or a monoclonal anti-CD68 (1/800 ZYMED, California, USA) antibody. Sections were washed, incubated in goat anti-rabbit peroxidase (1:500 in NGS) followed by fluorochromes TSA-plus cyanide 5 (1:50 in diluent).

For the triple immunohistochemistry sections were blocked in NGS and incubated overnight with a polyclonal anti-IL-11R α antibody at 1:700 (C-20; Santa Cruz). Sections were washed, incubated in goat anti-rabbit peroxidase (1:500 in NGS for 30 min) followed by fluorochromes TSA-plus cyanide 3 (1:50 in diluent). Sections were washed, antigen retrieval in citrate buffer and blocking in NGS was repeated before being incubated overnight with a polyclonal anti-PROKR1 antibody at 1:250 (Lifespan Biosciences, Atlanta, GA, USA). Sections were washed, incubated in goat anti-rabbit peroxidase (1:500 in NGS) followed by fluorochromes TSA-plus cyanide 5 (1:50 in diluent). Finally after further antigen retrieval and blocking steps, sections were incubated overnight with a monoclonal anti-IL-11 antibody at 1:100 (R&D Systems). Sections were washed, incubated in goat anti-mouse peroxidase (1:500 in NGS) followed by fluorochromes TSA-plus fluorescein (green) (1:50 in diluent).

All sections were washed, mounted in Permafluor and visualized using a laser-scanning microscope (meta confocal; Carl Zeiss, Jena, Germany).

Statistical analysis

Data are represented as mean \pm SEM and were analysed by t test or ANOVA using Prism 5.0c (Graph Pad, San Diego, CA, USA).

Results

PROK1 induces the expression and secretion of IL-11 via a calcineurin–NFAT dependent pathway in Ishikawa endometrial epithelial cells

In order to investigate the molecular mechanisms whereby PROK1 mediates the induction of IL-11, we made use of a human endometrial epithelial cell line, Ishikawa cells, stably expressing PROKR1 (Evans *et al.*, 2008). Wild-type Ishikawa and PROKR1 Ishikawa cells were treated with vehicle or 40 nM PROK1, for the times indicated in the figure legends. PROK1–PROKR1 stimulation in PROKR1 Ishikawa cells resulted in a significant increase in IL-11 mRNA expression which was maximal between 8 and 12 h ($P < 0.005$, Fig. 1A). No increase in IL-11 expression was observed in wild-type Ishikawa cells. All subsequent experiments were conducted in the PROKR1 Ishikawa cells. Secreted IL-11 protein, as measured by ELISA was maximal by 24 h ($P < 0.005$, Fig. 1B) after treatment with 40 nM PROK1. To investigate the signalling pathways mediating the role of PROK1 on IL-11 expression, PROKR1 Ishikawa cells were stimulated with vehicle or 40 nM PROK1 for 6 h in the presence or absence of the chemical inhibitors of $G_{q/11}$ (YM254890), a calcium chelator (EGTA), ERK (PD98059), NFAT (INCA-6) or calcineurin (CSA; cyclosporine A). Co-treatment of PROK1 cells with YM254890 ($P < 0.005$), EGTA ($P < 0.005$), PD98059 ($P < 0.05$), CSA ($P < 0.05$) and INCA-6 ($P < 0.005$) inhibited the expression of IL-11 mRNA (Fig. 1C). These data indicate that IL-11 expression in Ishikawa cells is regulated via PROK1–PROKR1 and downstream activation of the G_q –calcium–ERK–calcineurin–NFAT signal transduction pathway.

The IL-11 promoter region contains two putative NFAT binding sites

As IL-11 expression is regulated via the calcineurin–NFAT signal transduction pathway, we investigated if the IL-11 promoter sequence contained any NFAT binding sites. Using a stringent search engine (<http://www.genomatix.de/>) mapping the 5' flank region we identified two correctly orientated putative NFAT binding sites at 1.642 and 5.138 kb from the start codon for IL-11 (Fig. 1D).

PROK1 does not alter the expression of IL-11R α or GPI30 in human endometrial Ishikawa cells

Since we have shown that PROK1 increases the expression and secretion of IL-11 we subsequently investigated whether PROK1 altered the expression levels of the receptors for IL-11—namely IL-11R α and GPI30. Treatment of PROKR1 Ishikawa cells with 40 nM PROK1 had no effect on expression levels of IL-11R α or GPI30 at any of the time points investigated (Fig. 1E and 1F).

RCAN1-4 overexpression inhibits PROK1-induced expression of IL-11

Calcineurin signalling is known to be tightly regulated by the RCAN1-4, previously known as Down syndrome critical region gene 1 (Davies *et al.*, 2007). We investigated the temporal expression of RCAN1-4 in PROKR1 Ishikawa cells within the time frame of IL-11 activation. We have shown that treatment with 40 nM PROK1 maximally induces expression of RCAN1-4 mRNA by 1.5 h in a reciprocal manner to IL-11 (Fig. 2A) indicating that it could be a negative regulator of IL-11. Hence we investigated whether RCAN1-4 acts as a negative regulator for the PROK1 regulation of IL-11 expression and secretion. PROKR1 Ishikawa cells were infected with either empty adenovirus or adenovirus containing RCAN1-4 for 24 h prior to stimulation with vehicle or 40 nM PROK1 for times indicated in the figures. Overexpression of RCAN1-4 significantly reduced the PROKR1 mediated induction of IL-11 mRNA expression at 8 h (Fig. 2B; $P < 0.01$) and protein secretion at 12 h (Fig. 2C; $P < 0.05$) compared with cells infected with the control empty virus.

IL-11, IL-11R α and GPI30 expression levels in non-pregnant endometrium and first trimester decidua

RT–PCR analysis showed that IL-11 mRNA expression was significantly elevated in the late secretory phase compared with the proliferative phase of the menstrual cycle (Fig. 3A; $P < 0.05$) and was further elevated in first trimester decidua compared with pooled samples (early, mid, late and secretory phases) of non-pregnant endometrium (Fig. 3B; 48.8 ± 10.2 versus 0.36 ± 0.06 arbitrary units; $P < 0.005$). This is in agreement with the immunohistochemical observations of Dimitriadis *et al.* (2000) who also showed an increase in IL-11 staining during mid secretory endometrium and a further increase in late secretory endometrium. IL-11R α mRNA expression did not significantly change across the menstrual cycle in non-pregnant endometrium (Fig. 3C) but was significantly reduced in first trimester decidua compared with pooled samples of non-pregnant endometrium (Fig. 3D; 1.50 ± 0.11 versus 0.13 ± 0.03 arbitrary units; $P < 0.001$). This was in agreement with the published data by Karpovich *et al.* (2003). GPI30 mRNA expression was significantly elevated in the mid-late secretory phase compared with the proliferative phase of the menstrual cycle (Fig. 3E; $P < 0.005$; $P < 0.05$ respectively) but showed no significant change between first trimester decidua and pooled samples of non-pregnant endometrium (Fig. 3F). This was in agreement with the immunohistochemical observations of Classen-Linke *et al.* (2004) who also showed an increase in GPI30 staining during early-mid and late secretory endometrium.

Immunohistochemical expression of IL-11R α , GPI30, IL-11 and PROKR1

Localization of the site of expression of IL-11R α , GPI30, IL-11 and PROKR1 protein in endometrial tissue and first trimester decidua was investigated. Firstly, we have shown that IL-11R α (green channel) and GPI30 (red channel) expression co-localize (yellow channel) to the glandular epithelium and stromal compartments in mid secretory endometrium and first trimester decidua (Fig. 4A and B). Subsequently, we investigated the co-localization of IL-11 (green channel), IL-11R α (red

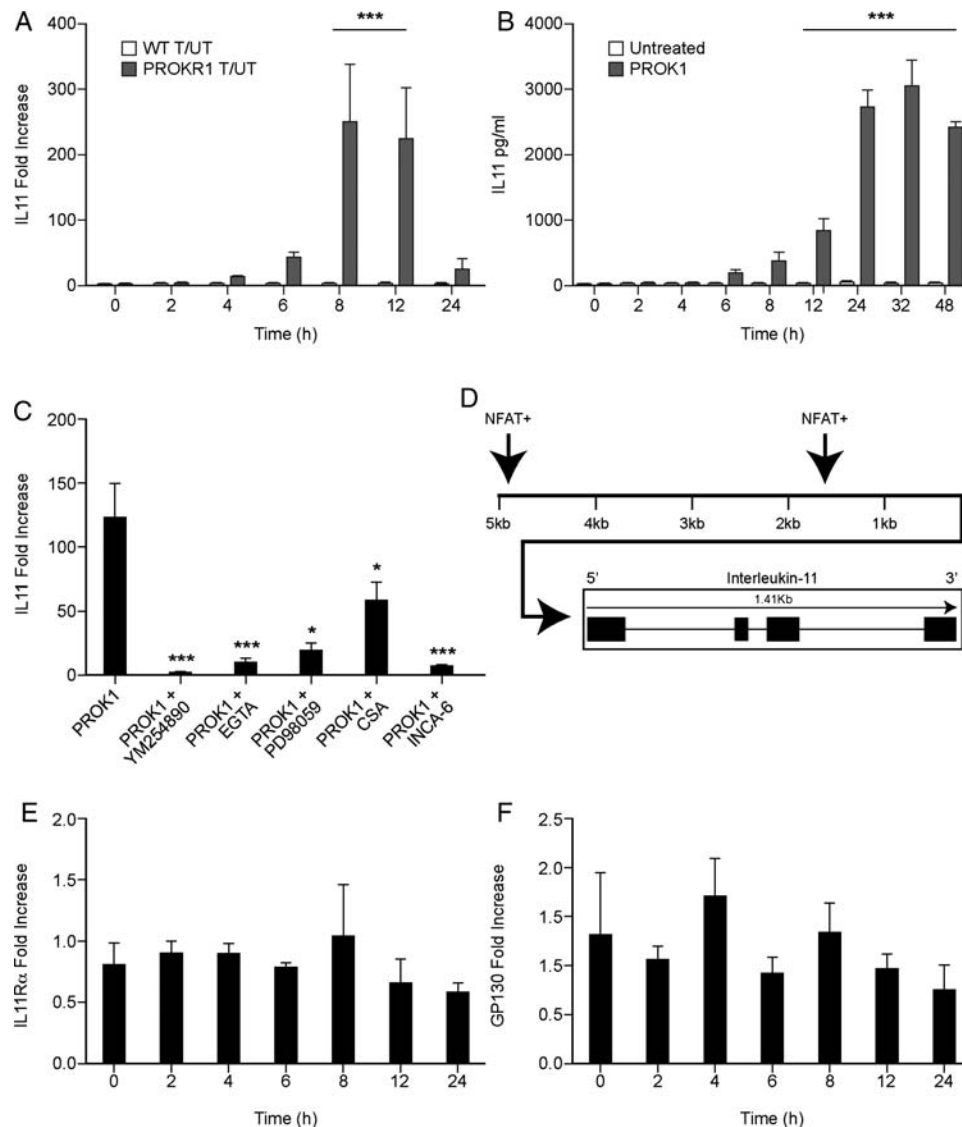


Figure 1 Prokineticin (PROK1) induces the expression and secretion of interleukin (IL)-11, but not interleukin receptor (IL-11R α) or glycoprotein receptor (GPI30), via a calcineurin/nuclear factor of activated T cells (NFAT) signalling pathway in PROKR1 Ishikawa cells. **(A)** PROKR1 Ishikawa cells, but not wild-type cells, treated with 40 nM PROK1 for 0–24 h showed a significant fold increase in the expression of IL-11 mRNA. **(B)** PROKR1 Ishikawa cells treated with 40 nM PROK1 for 0–48 h showed a significant increase in secretion of IL-11 protein compared with untreated cells. **(C)** The induction of IL-11 mRNA by PROK1 at 6 h could be inhibited by the use of a $G_{q/11}$ inhibitor (YM254890, 1 μ M), a calcium chelator (EGTA, 1.5 mM), an ERK inhibitor (PD98059 50 μ M) a calcineurin inhibitor (CSA 1 μ M) and a NFAT/calcineurin inhibitor (INCA-6, 40 nM). **(D)** Using a stringent search engine mapping the 5' flank region two correctly orientated NFAT binding sites at 1.642 and 5.138 kb from the start codon for IL-11 were identified <http://www.genomatix.de/>. **(E and F)** PROKR1 Ishikawa cells treated with 40 nM PROK1 for 0–24 h showed no significant fold change in the expression of IL-11R α or GPI30 mRNA. Data represent the mean \pm SEM of $n = 4$ –6 experiments. * $P < 0.05$ and *** $P < 0.005$.

channel) and PROKR1 (blue channel). In late secretory phase endometrium and first trimester decidua IL-11, IL-11R α and PROKR1 localize to the glandular epithelium and stromal cells as indicated by the purple channel (Fig. 4C and D). Immune cells make up a significant component of decidual tissue hence we investigated if IL-11R α co-localized with both uNK cells and macrophages. However, we found no co-localization between IL-11R α (red channel) and uNK cells (CD56—green channel, Fig. 4E) or macrophages (CD68—green channel, Fig. 4F) in first trimester decidua.

PROK1 induces the expression and secretion of IL-11 but not PROKR1 or GPI30 in first trimester decidua via a G_q - Ca^{2+} -ERK dependent pathway

We subsequently investigated whether PROK1 can regulate expression of IL-11 in first trimester decidua tissue explants. Treatment with 40 nM PROK1 resulted in an increase in IL-11 mRNA from 2–8 h (2.8 ± 0.66 -fold increase; $P < 0.05$) to 12–24 h (3.5 ± 0.64 -fold increase;

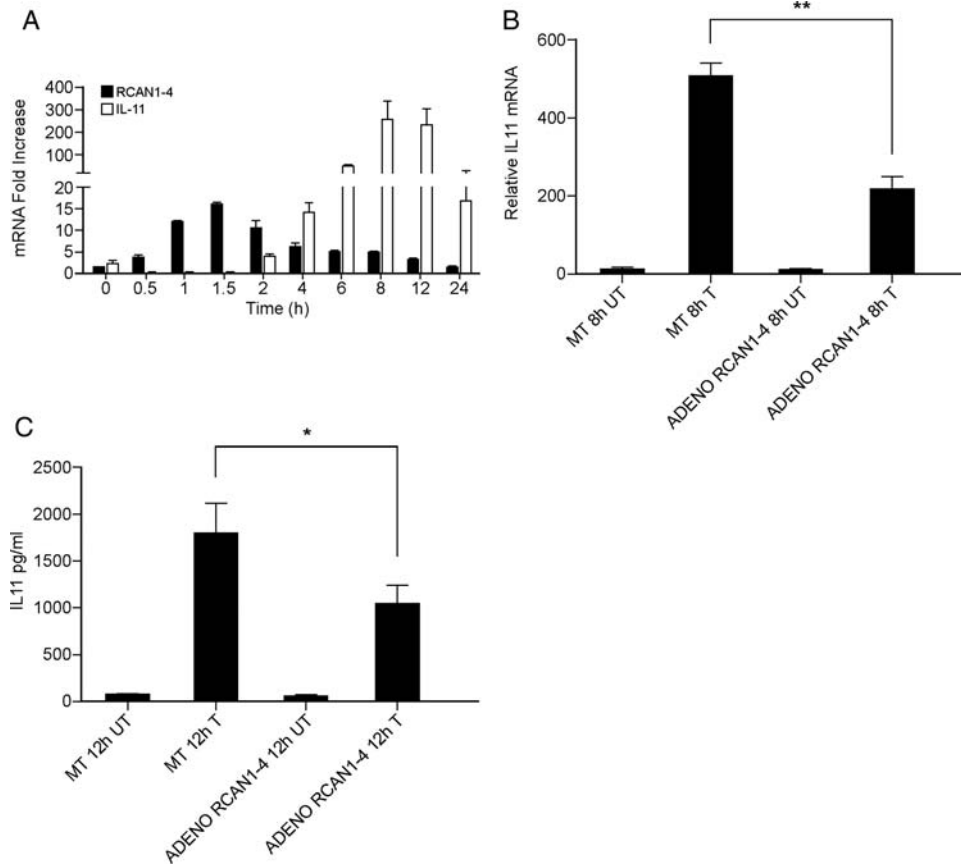


Figure 2 Regulator of calcineurin I isoform 4 (RCAN1-4) negatively regulates prokineticin (PROK1) induced expression of interleukin (IL)-11. **(A)** PROK1 Ishikawa cells treated with 40 nM PROK1 for 0–24 h showed a significant fold increase in the expression of RCAN1-4 and IL-11 mRNA with maximal levels for RCAN1-4 observed at 1.5 h and for IL-11 at 8 and 12 h. **(B and C)** PROK1 Ishikawa cells were infected with either empty adenovirus or RCAN1-4 adenovirus for 24 h. Cells were then treated with vehicle (UT) or 40 nM PROK1 (T) for 8 or 12 h. RCAN1-4 adenovirus compared with the empty vector adenovirus significantly reduced the PROK1 induced mRNA expression of IL-11 at 8 h (B) and protein secretion at 12 h (C). Data represent the mean \pm SEM of $n = 4$ experiments. * $P < 0.05$ and ** $P < 0.01$.

$P < 0.01$) (Fig. 5A). PROK1 induced expression of IL-11 mRNA at 24 h was significantly reduced by co-treatment of tissue explants with a $G_{q/11}$ inhibitor ($P < 0.05$), a calcium chelator ($P < 0.01$) or an ERK inhibitor ($P < 0.01$) (Fig. 5B). Treatment of first trimester decidua with 40 nM PROK1 had no effect on expression levels of IL-11R α or GPI30 (Fig. 5C and D). We confirmed that PROK1 regulates expression of IL-11 in first trimester decidua using a targeted PROK1 miRNA lentivirus. First trimester decidua was infected with one of three miRNA constructs targeting PROK1 (pLenti6/V5-EmGFP-hum-PROK1-72, -287 and a chained version-72_287) and a negative scrambled control lentivirus (as described in Evans *et al.*, 2009). Infection of first trimester decidua with either the pLenti6/V5-EmGFP-hum-PROK1-72 and -287 or the combination of 72 and 287 (-72_287) significantly reduced levels of both IL-11 mRNA and secreted IL-11 when compared with the negative scrambled control construct (Fig. 5E and F).

Discussion

PROK1, but not PROKR1, has previously been shown to be regulated across the menstrual cycle with increased expression in the mid

secretory phase (Battersby *et al.*, 2004). Furthermore, we have recently shown that both PROK1 and PROKR1 expression levels are significantly higher in first trimester decidua compared with non-pregnant endometrium (Evans *et al.*, 2008). Using epithelial cells stably expressing PROKR1 we have shown that PROK1 regulates genes known to have important roles within the endometrium during implantation and early pregnancy. These included LIF, COX-2, IL-6, IL-8 and IL-11 (Evans *et al.*, 2008). This highlighted the potential importance of PROK1–PROKR1 signalling within the endometrium and we focused this study on IL-11 due to its known importance in endometrial physiology.

IL-11 is crucial for controlling decidualization and implantation—both maternally and within the implanting embryo (Dimitriadis *et al.*, 2007; Guzeloglu-Kayisli *et al.*, 2009). IL-11 is thought to mediate these effects by controlling both cellular proliferation and differentiation (Mehler *et al.*, 1993; Du and Williams, 1994; Girasole *et al.*, 1994). This concurs with the observation that the IL-11R α $-/-$ female mice are infertile due to poor decidualization and (or) poor cellular differentiation (Bilinski *et al.*, 1998; Robb *et al.*, 1998). IL-11 has been shown to be produced and subsequently promote

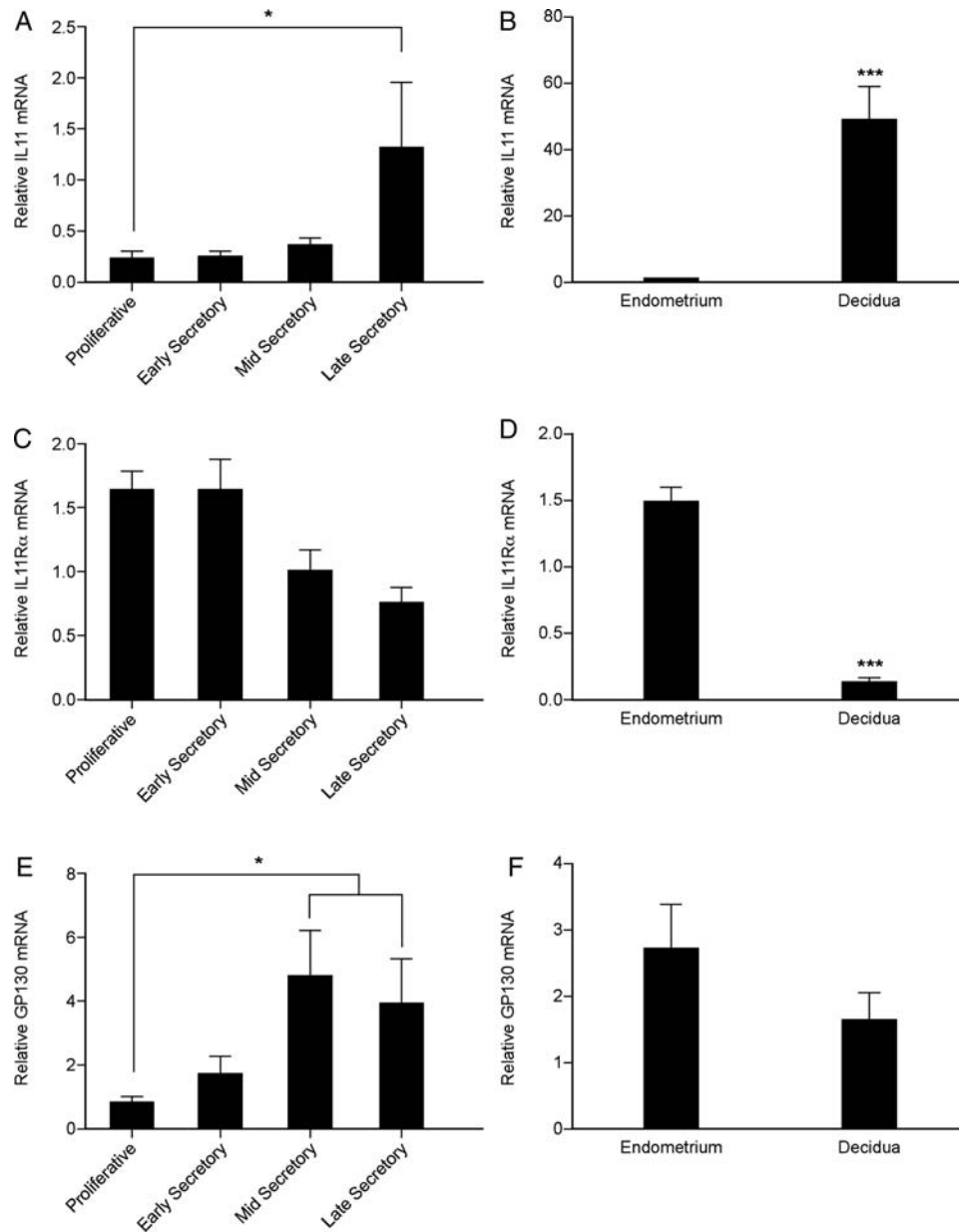


Figure 3 Interleukin (IL)-11, interleukin receptor (IL-11R α) and glycoprotein receptor (GPI30) mRNA expression levels in non-pregnant endometrium and first trimester decidua. **(A and B)** Temporal expression of IL-11 mRNA across the menstrual cycle showed a significant increase in the late secretory phase compared with the proliferative phase of the menstrual cycle. Temporal expression of IL-11 mRNA in first trimester decidua ($n = 25$) is significantly higher than in non-pregnant endometrium [grouped samples from all stages of the menstrual cycle ($n = 44$)]. **(C and D)** Temporal expression of IL-11R α mRNA across the menstrual cycle showed no significant change. Temporal expression of IL-11R α mRNA in first trimester decidua ($n = 25$) is significantly lower than in non-pregnant endometrium [grouped samples from all stages of the menstrual cycle ($n = 44$)]. **(E and F)** Temporal expression of GPI30 mRNA across the menstrual cycle showed a significant increase in the mid-late secretory phase compared with the proliferative phase of the menstrual cycle. Temporal expression of GPI30 mRNA in first trimester decidua ($n = 25$) is not significantly different when compared with non-pregnant endometrium [grouped samples from all stages of the menstrual cycle ($n = 44$)]. Proliferative $n = 15$, early secretory $n = 9$, mid secretory $n = 10$ and late secretory $n = 5$. Data represent the mean \pm SEM. * $P < 0.05$, *** $P < 0.005$.

progesterone-induced decidualization in human endometrial stromal cells (Dimitriadis et al., 2002). Furthermore relaxin and PGE₂ have both been shown to increase IL-11 levels in decidualized endometrial stromal cells (Dimitriadis et al., 2005a, b). These reports highlight the potential for both paracrine and autocrine actions within human

endometrial stromal cells in preparing the human endometrium for implantation. Furthermore, *in vitro* studies have shown that IL-11 promotes migration of human trophoblast cells, a process essential for placentation (Paiva et al., 2007). In human decidua, IL-11 has been shown to increase the expression of the metalloproteinase inhibitor

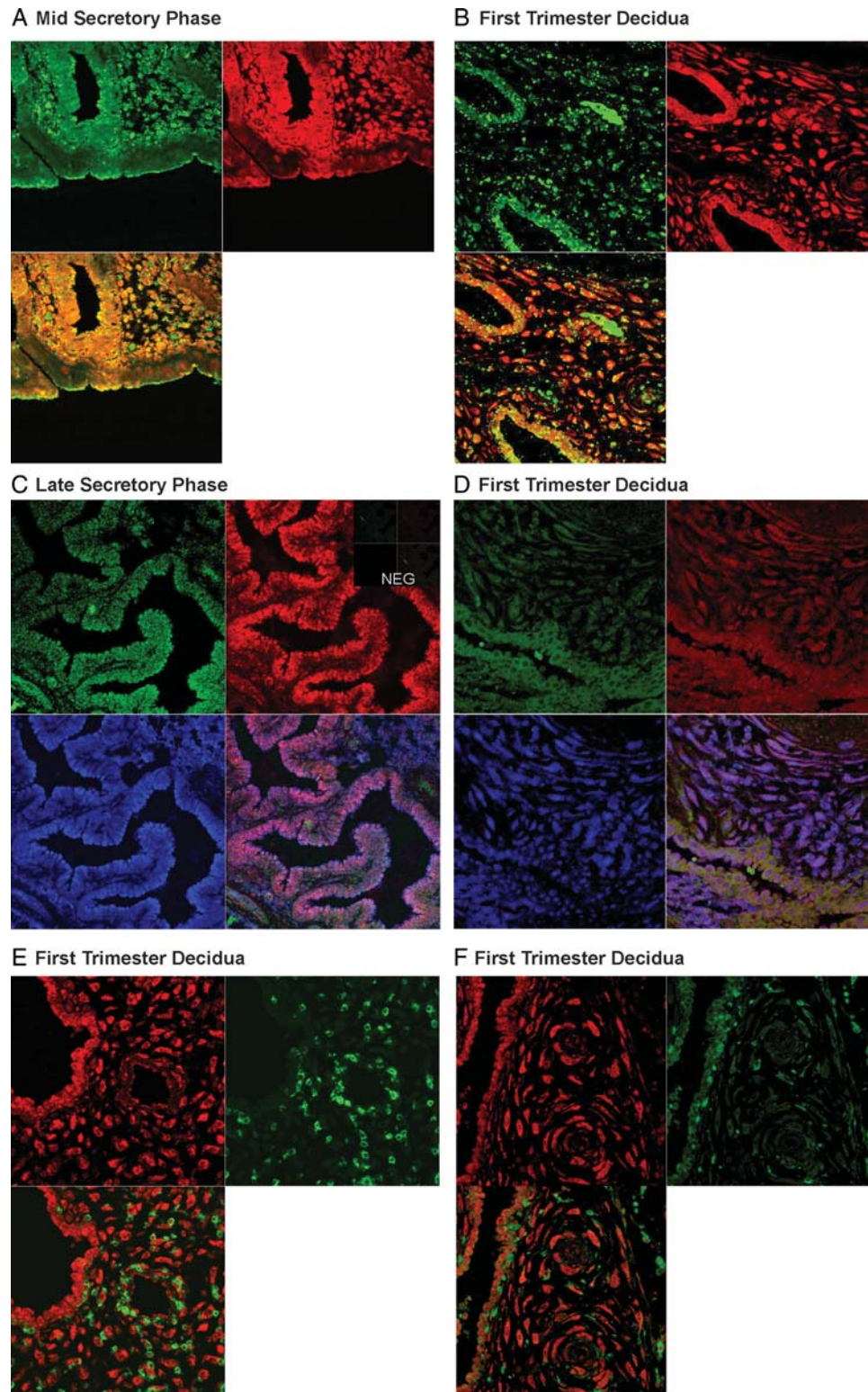


Figure 4 Immunohistochemical localization of Interleukin receptor (IL-11R α), glycoprotein receptor (GPI30), interleukin (IL-11) and prokineticin receptor (PROKR1) in non-pregnant endometrium and first trimester decidua. (A and B) IL-11R α (green channel) and GPI30 (red channel) co-localize (yellow) to glandular epithelium and stromal cells in mid secretory endometrium and first trimester decidua ($n = 4$; representative sections shown). (C and D) IL-11 (green channel), IL-11R α (red channel) and PROKR1 (blue channel) co-localize (purple channel) to glandular epithelium and stromal cells in late secretory endometrium and first trimester decidua ($n = 3$; representative sections shown, inset, negative control incubated with control IgG). (E) IL-11R α (red channel) and CD56 (green channel) and (F) IL-11R α and CD68 (green channel) do not co-localize in first trimester decidua ($n = 4$; representative sections shown).

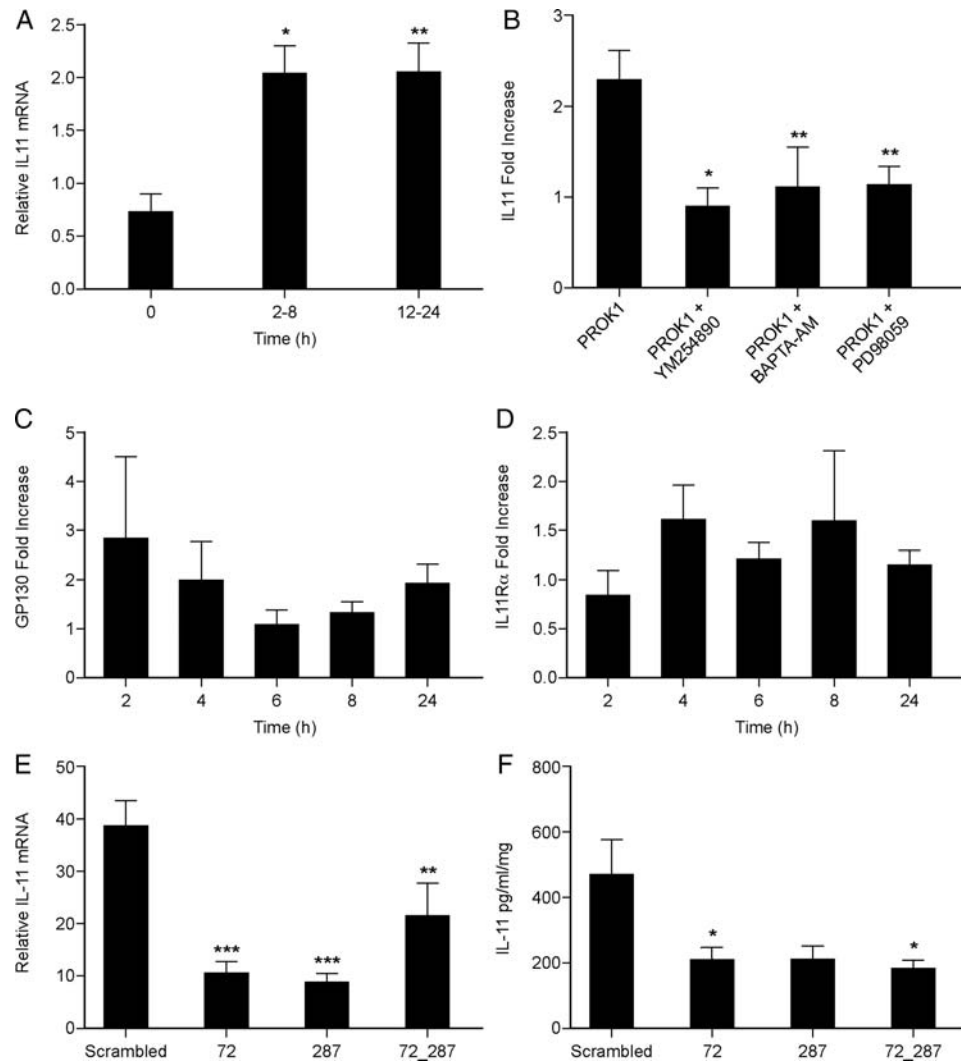


Figure 5 Prokineticin (PROK1) induces the expression and secretion of interleukin (IL-11) but not interleukin receptor (IL-11R α) or glycoprotein receptor (GPI30) in first trimester decidua. **(A)** First trimester decidua explants treated with 40 nM PROK1 for 0–24 h showed a significant fold increase in the expression of IL-11 mRNA. **(B)** The induction of IL-11 mRNA by PROK1 treatment for 24 h could be significantly inhibited by the use of a guanine nucleotide-binding protein ($G_{q/11}$) inhibitor (YM254890, 1 μ M), a calcium chelator (BAPTA-AM, 50 μ M) and an ERK inhibitor (PD98059, 50 μ M). **(C and D)** First trimester decidua explants treated with 40 nM PROK1 showed no significant fold change in the expression of IL-11R α or GPI30 mRNA. First trimester decidua were infected with miRNA constructs targeting PROK1 (72, 287 and 72_287) or a scrambled control sequence for 72 h. IL-11 mRNA expression **(E)** and protein secretion **(F)** were both significantly reduced by all miRNAs targeted to PROK1 compared with the scrambled control. Data represent the mean \pm SEM of $n = 3$ –8 experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$.

α 2-macroglobulin in order to control normal placentation (Bao et al., 2006). However, the molecular mechanism regulating IL-11 and its autocrine/paracrine role in human uterine function of early pregnancy is unclear.

In the present study, we report that PROK1 induces the expression and secretion of the cytokine IL-11 in cultured endometrial epithelial cells and first trimester decidua explants. Furthermore we have shown that the PROK1–PROKR1 induction of IL-11 is mediated via the calcineurin signalling pathway in a $G_{q/11}$, calcium and ERK dependent manner as shown schematically in Fig. 6. Calcium dependent activation of calcineurin results in dephosphorylation of cytoplasmic NFAT, leading to NFAT translocation to the nucleus enabling it to

activate NFAT regulated gene transcription. Using a highly stringent search we have identified two correctly orientated putative NFAT binding sites within the IL-11 promoter. Moreover, using a specific inhibitor, INCA-6, which is known to block the interaction between calcineurin and NFAT (Roehrl et al., 2004), we have shown that inhibiting NFAT signalling can block PROK1–PROKR1 mediated expression of IL-11.

RCAN1-4 is an endogenous modulator of the calcineurin signalling pathway. Previous studies in our laboratory have identified that PROK1 can induce expression of *RCAN1* by gene array analysis (Evans et al., 2008). RCAN1-4 is known to bind to calcineurin and previous studies have shown that overexpression of RCAN1-4

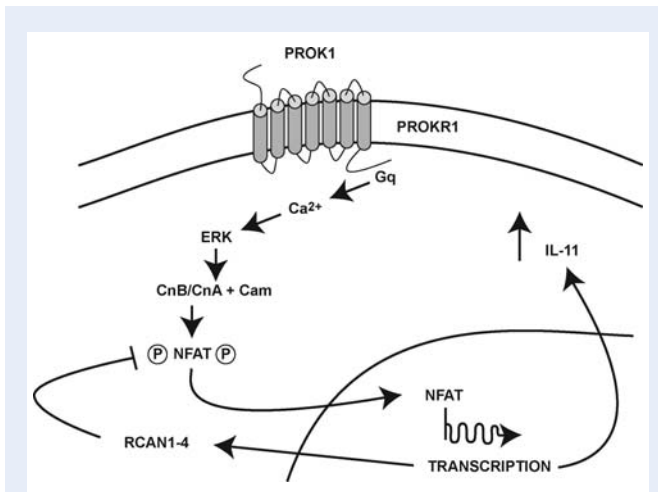


Figure 6 Schematic representation of Prokineticin (PROK1) induction of regulator of calcineurin I isoform 4 (RCAN1-4) and interleukin (IL-11). Activation of the PROKR1 by PROK1 results in the induction of IL-11 expression. This occurs via coupling to $G_{q/11}$ protein. This results in an intracellular increase in calcium which activates calcineurin and subsequently dephosphorylates cytoplasmic NFAT. This allows NFAT to migrate to the nucleus and bind to NFAT binding motifs in the promoter of IL-11 and induce its transcription. PROK1 also up-regulates RCAN1-4 expression which acts as a negative regulator and reduces the level of IL-11 transcription by binding to calcineurin and hence inhibiting NFAT dephosphorylation. $G_q = G_q$ protein alpha subunit, Ca^{2+} = intracellular ionized calcium, ERK = extracellular signal-regulated kinase, Cam = calmodulin, CnA = calcineurin catalytic subunit, CnB = calcineurin regulatory subunit and NFAT = nuclear factor of activated T-cells.

results in an inhibition of calcineurin activation of NFAT (Minami *et al.*, 2004; Chan *et al.*, 2005). Here, we have shown that RCAN1-4 is temporally regulated in a reciprocal manner to expression of IL-11 by PROK1–PROKR1. Moreover we have shown that overexpression of RCAN1-4 leads to a reduction in PROK1 induced IL-11 indicating that RCAN1-4 is acting as a negative regulator of PROK1–PROKR1 mediated IL-11 signalling as summarized in Fig. 6.

In order to confirm the physiological relevance of our data, we investigated the effect of PROK1 on expression of IL-11 in first trimester decidua tissue explants. In accordance with our observation using the PROKR1 Ishikawa cell line, we showed that IL-11 in first trimester decidua is also regulated by PROK1, since treatment of decidua tissue with exogenous PROK1 increased IL-11 induction. We further confirmed PROK1 regulation of IL-11 in first trimester decidua using miRNA constructs targeted to PROK1. Ablation of endogenous PROK1 in first trimester decidua inhibited IL-11 expression confirming that PROK1 regulates the basal expression and secretion of IL-11.

IL-11 is a secreted cytokine which is known to signal via its receptors, IL-11R α and GPI30; hence we investigated the potential for autocrine or paracrine signalling by localizing the sites of protein expression of GPI30, IL-11R α , IL-11 and PROKR1. Immunohistochemistry showed that both IL-11R α and GPI30 co-localized in glandular epithelium and stromal compartments of both endometrium and first trimester decidua. This was in agreement with the published data

by Karpovich *et al.* (2003) and Classen-Linke *et al.* (2004). We have also shown co-localization of IL-11, IL-11R α and PROKR1 in glandular epithelium and stroma suggesting the potential for PROK1–PROKR1 to regulate IL-11 expression in both compartments during endometrial receptivity and early pregnancy in an autocrine–paracrine manner. Immune cells make up a significant component of decidua and IL-11 has been shown to be important in the differentiation of uNK cells in decidua (Ain *et al.*, 2004). However, we saw no co-localization between IL-11R α and uNK cells or macrophages in decidua. This suggests that the role of IL-11 in regulating uNK cell differentiation is by an indirect non-paracrine mechanism.

Subsequently, we have shown that both IL-11 and GPI30 mRNA expression are elevated in the late secretory phase of the menstrual cycle whereas IL-11R α shows no change. IL-11 is further elevated in first trimester decidua but conversely IL-11R α is significantly lower compared with non-pregnant endometrium. This is likely to be a compensatory mechanism accounting for the large increase in IL-11, as seen in first trimester decidua and acts to control IL-11's signalling potential and to maintain specificity between other GPI30 signalling cytokines. GPI30 showed no difference between non-pregnant endometrium and first trimester decidua which is in agreement with Classen-Linke *et al.* (2004).

Taken together, our data suggest that PROK1–PROKR1 signalling to IL-11 could play a vital role in decidualization, implantation and maintenance of pregnancy. IL-11 is known to enhance progesterone mediated decidualization and that relaxin and PGE₂ enhance IL-11 expression (Dimitriadis *et al.*, 2002, 2005a, b). Therefore PROK1–PROKR1 could increase IL-11 expression and secretion directly during decidualization in a complementary manner. IL-11 induction via PROK1–PROKR1 may affect blastocyst implantation by increasing the expression of the metalloproteinase inhibitor, α 2-macroglobulin, or via IL-11 mediated migration of trophoblast cells (Bao *et al.*, 2006, Paiva *et al.*, 2007). Recently, it has been shown that IL-11 can increase the adhesion of human endometrial epithelial cells to both fibronectin and collagen IV and that IL-11 could increase adhesion of trophoblast cells to endometrial cells (Marwood *et al.*, 2009) again suggesting IL-11 could be regulating the process of implantation.

In conclusion, our data has mapped out for the first time, the intricate molecular mechanisms whereby IL-11 is regulated in endometrial epithelial cells and first trimester decidua by PROK1. Our findings together with others suggest that PROK1 may be important in regulating decidualization and human fertility via the expression of IL-11. Dys-regulated expression and/or signalling of the prokineticin pathway may contribute to female infertility.

Acknowledgements

We thank Sharon Donaldson and Catherine Murray for consenting patients and tissue collection.

Funding

This study was supported by MRC core funding to H.N.J. (U1276.00.004.00002.03).

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- Submitted on May 5, 2009; resubmitted on September 16, 2009; accepted on September 29, 2009